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(54) Title: PEPTIDES FOR USE IN THE IMMUNOTHERAPY OF RENAL CELL CARCINOMA

(57) Abstract: The present invention relates to peptides for use in immunotherapy of tumors, in particular renal cell carcinoma's. The peptides are derived from the amino acid sequence of a renal cell carcinoma-associated antigen, called the G250 protein. A particularly preferred peptide has the amino acid sequence: HLSTAFARV. The peptides of the invention may be formulated into pharmaceutical composition for administration to humans or animals. Alternatively, the peptides may be administered to a human or animal in conjunction with an antigen presenting cell, or they may be encoded on a gene therapy agent to be expressed by the human or animal.

Peptides for use in the immunotherapy of renal cell carcinoma

Field of the invention

The present invention relates to peptides that can be used to elicit an immune response in a human or animal. In particular, the invention relates to (oligo)peptides that can be used to elicit an immune response in a human or animal against a tumor, and specifically against an immunogenic tumor. More in particular, the invention relates to (oligo)peptides that can be used in the immunotherapy of renal cell carcinoma.

Background of the invention

According to one way of classifying tumors, in particular human carcinomas, a distinction is made between immunogenic and non-immunogenic tumors. Immunogenic tumors can be described as tumors that express certain antigens on their surface. Examples thereof include melanoma, renal cell carcinoma or other cancers of the kidneys, as well as cancer(s) of the prostate, the head and/or neck, the colon, the stomach, and/or the bladder. The finding that immunogenic tumors express specific antigens on their surface has opened up the possibility of treating such tumors using immunological techniques. So far, this "immunotherapy approach" has focused on the so-called "humoral" immune response, in which the object is to provide antibodies that are specifically directed against the antigens present on the tumors. This is usually carried out using *in vitro* techniques known per se for generating polyclonal or monoclonal antibodies, such as immunisation of a mammal with the tumor antigen, or via *in vitro* production using antibody-producing hybridomas.

According to this humoral approach, therapeutic treatment generally involves administration of the antibodies thus obtained to a patient. Reference is for instance made to the international application WO 93/18152, which describes a gene called the *MN*-gene, the expression of which was found to be strongly correlated with tumorigenicity (vide also WO 95/3460, which gives the correct sequence for the MN-gene). WO 93/18152 also describes specific antibodies directed against the proteins or polypeptides encoded by the *MN*-gene, and various diagnostic and therapeutic uses thereof, including treatment of neoplastic and pre-neoplastic diseases.

By contrast, the present invention is not based on a "humoral" immune response, but on a "cellular" immune response. Generally, this approach involves administering to a patient an antigen that is capable of *in vivo* eliciting an immune response against (the antigens present on) the tumor cells, upon which the immune system of the patient is relied upon to provide the

actual response that "attacks" the tumor, for instance by generating antibodies against the antigen/tumor and/or cytotoxic T lymphocytes directed against the tumor (cells).

In principle, this cellular approach offers a number of advantages compared to the humoral approach. For one, the treatment of the tumor will generally involve only a limited number of administrations ("*vaccinations*") with the antigen - usually only one or more priming administrations followed by one or more booster administrations - upon which the body will in essence continuously provide the desired immune response against the tumor. By contrast, humoral immune therapy may involve repeated administrations of the antibodies, e.g. until the tumor has disappeared.

Another advantage of the cellular approach is that tumor cell death may (also) be induced by the so-called "bystander"-effect, by which is meant that antigen-negative cells may die through the release of cytotoxic substances that are released from an adjacent antigen-positive cell that is recognised/attacked by the cellular immune response.

As will be clear from the above, the cellular approach requires the use of tumor specific antigens that can be suitably administered to a patient, so as to generate the desired immune response. Thus, one of the main objects of the present invention is to provide such antigens, as well as pharmaceutical compositions containing such antigens.

In this respect, besides the *MN*-specific antibodies mentioned above, WO 93/18152 also describes the use of proteins or polypeptides encoded by the *MN*-gene, as well as fragments thereof, as or in vaccines for inducing protective immunity against neoplastic disease and/or a dampening effect upon tumorigenic activity.

According to WO 93/18152, for this purpose, preferably a genetically engineered MN-protein is used; and/or the MN-protein is incorporated into a multimeric structure, bonded to a protein carrier, or used as a fusion protein, for instance with S-glutathion transferase (which fusion is referred to in WO 93/18152 as "*pGEX-3X-MN*"). Thus, the method of WO 93/18152 generally involves the use of large immunogenic proteins, which for the reasons outlined below cannot be used in the present invention.

Recently, a widely expressed RCC-associated antigen called the G250 protein has been identified. Also, using a monoclonal antibody (mAb) directed against this G250 protein, it was shown that this RCC-associated antigen is expressed on the cell surface of approximately 85% of RCC but not normal kidney tissue (Oosterwijk, E. et al., Int. J. Cancer, 38: 489-494, 1986.)

In addition, G250 expression has been detected on the cell surface of colon-, ovarian- and cervical carcinomas. Analysis of normal tissues indicated that the reactivity of the G250

mAb is limited to some gastric mucosal cells and cells of the larger bile ducts. The staining observed in these normal tissues is relatively weak and cytoplasmic in nature. Clinical studies in RCC patients demonstrated exclusive targeting of radiolabeled mAb G250 to RCC (Oosterwijk, E., et al., Semin.Oncol., 22: 34-41, 1995.)

Recently, the cDNA encoding the RCC-associated antigen recognised by a mAb G250 was identified (Oosterwijk, E., et al., Int.J Cancer, 38: 489-494, 1986.) The deduced amino acid sequence of the G250 antigen showed that the G250 protein is a transmembrane protein identical to the above-mentioned tumor-associated antigen MN/CA IX identified in cervical carcinoma. Sequence comparison of the G250/MN/CA IX gene with RCC-derived cDNA of G250 demonstrated that the G250/MN/CA IX protein in RCC is non-mutated.

However - for reasons that will become clear hereinbelow - the full MN or G250 protein cannot be used for immune therapy of cancer via the cellular approach. A specific purpose of the invention was therefore to provide antigens based upon the G250 protein suitable for use in the treatment of cancer via the cellular approach.

Description of the invention

In the research leading up to the present invention, the immunogenicity of the RCC-associated antigen G250 was investigated using the reversed immunology approach. By this approach, a number of possible antigens based upon the amino acid sequence of the G250 protein were developed. In particular, it was found that of these, the G250 amino acid sequence from 254 to 262 is an HLA-A2.1-restricted CTL epitope that is both naturally processed and immunogenic, and thus can be used in the immune therapy of cancers, in particular of cancers expressing the G250 protein.

In a first aspect, the invention relates to an (oligo)peptide containing a sequence of 6-15, preferably 10-8, more preferably 9, contiguous amino acids from the amino acid sequence of SEQ ID no.1. Such (oligo)peptides, that in essence comprise a small part of the amino acid

molecules, such as e.g. peptides (or polypeptides). Such peptides comprising a sequence of 6-15, preferably 10-8, more preferably 9, contiguous amino acids from the amino acid sequence of SEQ ID no.1 are by definition not the native human G250 because the peptides do not comprise all of the amino acid sequence of SEQ ID no.1. Preferably the peptides comprising the oligopeptides of SEQ ID no.1 do not contain more than 90, 70, 50, 40, 30, 25, 20, 15 or 10% of the amino acid sequence of SEQ ID no1. More preferably, the peptides comprising the oligopeptides of SEQ ID no.1 do not contain more than 100, 75, 50, 40, 30, 25, 20, 15 or 10 contiguous amino acids from the amino acid sequence of SEQ ID no.1. The practical purposes the (oligo)peptides of SEQ ID no.1 may be part of larger molecules. The larger molecules may be linear polypeptides comprising the oligopeptides of SEQ ID no.1, or, alternatively the larger molecule may be any type of carrier molecule or structure to which the (oligo)peptides of SEQ ID no.1 are covalently (cross)linked by any means known per se for (cross)linking of peptides.

The invention also relates to certain variants/analogues of the (oligo)peptides of SEQ ID no.1, which will be further defined hereinbelow. Also, hereinbelow, the (oligo)peptide(s) of SEQ ID no.1 and the variants/analogues thereof will be collectively referred to as "*oligomer(s)*".

In a second aspect, the invention relates to a pharmaceutical composition containing an (oligo)peptide of SEQ ID no.1 or a variant or analogue thereof.

In a further aspect, the invention relates to the use of an (oligo)peptide of SEQ ID no.1 or a variant or analogue thereof in the preparation of a composition for the prevention and/or treatment of immunogenic tumors, and in particular of melanoma and renal cell carcinoma.

Further aspects, embodiments and advantages of the invention will become clear from the description given hereinbelow.

According to a preferred embodiment, the (oligo)peptide of SEQ ID no.1 is an (oligo)peptide comprising 6-15, preferably 8-10, and more preferably 9 amino acid residues that comprises one of the amino acid sequences of Table I (or - in case the (oligo)peptide of SEQ ID no.1 has less than 9 amino acid residues - a corresponding part thereof).

More preferably, the (oligo)peptide of SEQ ID no.1 is an (oligo)peptide comprising 6-15, preferably 8-10, and more preferably 9 amino acid residues that comprises one of the preferred amino acid sequences indicated in **bold** in Table I (or - in case the (oligo)peptide of SEQ ID no.1 has less than 9 amino acid residues - a corresponding part thereof).

TABLE I: (oligo)peptides of the invention and their position in the sequence of SEQ ID no 1.

(The amino acid residues are indicated according to the "one-letter"code. The preferred (oligo)peptides are indicated in **bold**.).

LLIPAPAPGL	G250:12-21
LLSLLLLMPV	**G250:26-35**
SLKLEDLPTV	G250:107-116
QLAAFCPAL	G250:169-177
ELLGFQLPPL	G250:181-190
LLGFQLPPL	G250:182-190
HLSTAFARV	**G250:254-262**
GLAVLAAFL	**G250:271-279**
QLLSRLEEI	G250:291-299
RVIEASFPA	G250:384-392
CLAAGDILAL	G250:409-418
ILALVFGLL	G250:415-423
GLLFAVTSV	**G250:421-429**

More preferably, according to this embodiment, the (oligo)peptide of SEQ ID no.1 is one of the (oligo)peptides of Table I, and in particular one of the four preferred (oligo)peptides of Table I, i.e. LLSLLLLMPV (G250:26-35); HLSTAFARV (G250:254-262); (GLAVLAAFL G250:271-279) and/or GLLFAVTSV (G250:421-429). According to a particularly preferred embodiment of the invention, the (oligo)peptide of SEQ ID no.1 is an (oligo)peptide comprising 6-15, preferably 8-10 amino acid residues that comprises the amino acid sequence of Formula I (or - in case the (oligo)peptide of SEQ ID no.1 has less than 9 amino acid residues - a corresponding part thereof):

HLSTAFARV (I).

According to the most preferred embodiment of the invention, the (oligo)peptide of SEQ ID no.1 is the nonapeptide of Formula I.

Preferably, the (oligo)peptides of SEQ ID no.1 are such that - upon suitable administration to the body of a human or animal (mammal), e.g. as described hereinbelow - they can generate or elicit an immune response in the human or animal (mammal), e.g. against (at least) the (oligo)peptide. Preferably, this immune response is a "*significant*" immune response, by which hereinbelow is generally meant a response that leads to a detectable change in the body of the human or animal to which the (oligo)peptide is administered.

Usually, this will be a detectable immune response against the (oligo)peptide, such as the generation of antibodies against the (oligo)peptide or another immune response that occurs when a body - or a part, organ or tissue thereof - is exposed to an antigen.

It will be clear that such an (significant) immune response will usually not be directed only against the (oligo)peptides of SEQ ID no.1, but also against proteins or peptides that contain such an (oligo)peptide as part of their amino acid sequence, as well as against structures, cells or tissues that contain, carry and/or express - e.g. on their surface(s) - peptides or proteins containing such an (oligo)peptide, such as (the cells of) the tumor to be treated.

Generally, whether an "oligomer" of the invention – i.e. an (oligo)peptide of SEQ ID no.1 or a variant or analogue thereof as defined hereinbelow - provides a significant immune response can for instance be determined using an immunological assay or an immunological detection technique known per se. Such an assay or detection technique may for instance be an antigen-based assay or detection technique, in which for instance the pertinent oligomer, another oligomer of the invention, and/or the protein or polypeptide of SEQ ID no.1 or an antigenic part thereof may be used as the antigen.

Examples of suitable immunological assays or detection techniques include, but are not limited to, blotting techniques such as Western blotting, ELISAs and RIAs, etc., for which reference is made to the standard handbooks, as well as for instance WO 93/18152.

This assay or detection technique is usually applied to a suitable biological sample or fluid obtained from the patient, such as blood, lymph fluid and/or a tissue sample, including but not limited to a sample obtained from/through a biopsy. Such a sample may for instance be obtained after suitable administration of the oligomer, e.g. as described below. The results obtained for this sample may then be compared to results obtained - i.e. using the same immunological assay - for a similar sample obtained prior to administration of the oligomer, to determine whether a significant immune response has been generated or not. The assay or detection technique may also be a quantitative technique, providing comparative data on the immune response generated by different oligomers of the invention.

More preferably, the (oligo)peptides of SEQ ID no.1 are such that they provide a "*therapeutically effective*" immune response, by which is meant an immune response that can attack or destroy a tumor present in the body of a patient, or at least prevent or limit the (further) growth and/or the spread of a tumor (i.e. within the same part or organ of the body and/or to other parts or organs of body), including but not limited to recidivism and/or metastasis.

It will be clear to the skilled person that certain variants or analogues of the (oligo)peptides of SEQ ID no.1 may also be suitable for use in the invention, and these will be referred to hereinbelow as "*(oligo)peptide analogues*". Preferably, these (oligo)peptide analogues are such that - upon suitable administration to the body of a human or animal (mammal), e.g. as described hereinbelow - they can generate or illicit in the human or animal (mammal) an immune response, preferably a significant immune response, against the (oligo)peptide analogue, and most preferably also against the corresponding (oligo)peptide of SEQ ID no.1. Most preferably, the (oligo)peptide analogues will provide a therapeutically effective immune response as defined hereinabove. Generally, the (oligo)peptide analogues of the invention will have essentially the same amino acid sequence as the (corresponding) (oligo)peptides of SEQ ID no.1; and only differ therefrom in at most 3, preferably at most 2, and most preferably only 1 amino acid residue(s), e.g. by an amino acid substitution, deletion, insertion and/or addition (hereinbelow collectively referred to as amino acid "*alterations*") in which an amino acid insertion, addition or deletion is counted as a difference in a single one amino acid residue.

Preferably, the (oligo)peptide analogues differ from the (oligo)peptides of SEQ ID no.1 by at most 3, preferably at most 2, and preferably only one amino acid substitution(s). In principle, any amino acid from any (oligo)peptide of SEQ ID no.1 can be replaced by any amino acid residue, as long as the analogue thus obtained is still capable of generating an immune response, in particular a significant immune response, more in particular a protective immune response. For instance, such a substitution may comprise one or more "conservative" amino acid substitutions, as are well known in the art. Conservative amino acid substitutions refer to the interchangeability of residues having similar side chains. For example, a group of amino acids having aliphatic side chains is glycine, alanine, valine, leucine, and isoleucine; a group of amino acids having aliphatic-hydroxyl side chains is serine and threonine; a group of amino acids having amide-containing side chains is asparagine and glutamine; a group of amino acids having aromatic side chains is phenylalanine, tyrosine, and tryptophan; a group of amino acids having basic side chains is lysine, arginine, and histidine; and a group of amino acids having sulfur-containing side chains is cysteine and methionine. Preferred conservative amino acids substitution groups are: valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, and asparagine-glutamine.

For the preferred (oligo)peptides mentioned in Table I, some particularly preferred amino acid substitutions include those mentioned in Table II.

Table II: preferred amino acid substitutions in the (oligo)peptides of Table I

(oligo)peptides		a.a. (position)	substitution
LLIPAPAPGL	G250:12-21	L (2)	M
		L (10)	V, P, A, F, W or M
LLSLLLLMPV	G250:26-35	L (2)	M
		V (10)	L, P, A, F, W or M
SLKLEDLPTV	G250:107-116	L (2)	M
		V (10)	L, P, A, F, W or M
QLAAFCPAL	G250:169-177	L (2)	M
		L (9)	V, P, A, F, W or M
ELLGFQLPPL	G250:181-190	L (2)	M
		L (10)	V, P, A, F, W or M
LLGFQLPPL	G250:182-190	L (2)	M
		L (9)	V, P, A, F, W or M
HLSTAFARV	G250:254-262	L (2)	M
		V (9)	L, P, A, F, W or M
GLAVLAAFL	G250:271-279	L (2)	M
		L (9)	V, P, A, F, W or M
QLLSRLEEI	G250:291-299	L (2)	M
		I (9)	L, P, V, A, F, W or M
RVIEASFPA	G250:384-392	V (2)	M
		A (9)	L, P, V, A, F, W or M
CLAAGDILAL	G250:409-418	L (2)	M
		L (10)	V, P, A, F, W or M
ILALVFGLL	G250:415-423	L (2)	M
		L (10)	V, P, A, F, W or M
GLLFAVTSV	G250:421-429	L (2)	M
		V (9)	L, P, A, F, W or M

If the (oligo)peptide analogue is provided by one or more amino acid deletions, insertions or additions, these are most preferably such that total number of amino acid residues in the (oligo)peptide analogue is 6-15, preferably 8-10, and most preferably 9. Thus, an (oligo)peptide analogue may for instance comprise a sequence of 18 contiguous amino acid residues from the sequence of SEQ ID no.1, from which three amino acid residues have been deleted.

The one or more amino acid alterations that lead to the (oligo)peptide analogues of the invention may be at any amino acid position(s) of any (oligo)peptide of SEQ ID no.1, and may be at adjacent positions and/or at non-adjacent positions. Preferably, the one or more amino acid substitutions, deletions and/or insertions occur near one or both of the termini of the corresponding (oligo)peptide(s) of SEQ ID no.1, i.e. in the three, preferably two amino acid residues at the C'-end and/or at the N'-end thereof, although the invention is not limited

thereto. Also, the one or more amino acid additions may be at one or at both ends of the (oligo)peptide of SEQ ID no.1. Preferably, the (oligo)peptide analogue is derived from one of the preferred (oligo)peptides of SEQ ID no.1 (i.e. an (oligo)peptide of SEQ ID no.1 that at least comprises one of the amino acid sequences of the above Table I, or part thereof), and in particular from one of the more preferred (oligo)peptides of SEQ ID no.1; e.g. by one or more of the amino acid alterations mentioned above. Such (oligo)peptide analogues may again comprise 6-15 amino acid residues, and preferably comprise 8-10, more preferably 9 amino acid residues. More preferably, the (oligo)peptide analogue is derived from one of the (oligo)peptides of Table I, and in particular from one of the preferred (oligo)peptides of Table I; e.g. by one or more of the amino acid alterations mentioned above. In such (oligo)peptide analogues, the one or more amino acid alterations are such that the total number of amino acid residues in the (oligo)peptide analogue is 10-8, and more preferably 9. Even more preferably, these (oligo)peptide analogues comprise one of the nonapeptides of Table I, and in particular one of the preferred nonapeptides of Table I, in which at most 3, preferably at most 2, and most preferably at most 1 amino acid residue have/has been replaced by another amino acid residue.

According to a particularly preferred embodiment, the (oligo)peptide analogue is derived from the nonapeptide of Formula I. Again, in such an (oligo)peptide analogue, the one or more amino acid alterations are such that the total number of amino acid residues in the (oligo)peptide analogue is 10-8, and more preferably 9. More preferably, such an (oligo)peptide analogue comprises the amino acid sequence of Formula I, in which at most 3, preferably at most 2, and most preferably at most 1 amino acid residue(s) have/has been replaced by another amino acid residue.

Some preferred substitutions in the amino acid sequence of Formula I to provide an (oligo)peptide analogue of the invention comprise L (2) to M; and/or V (9) to L, P, A, F, W or M

It is envisaged that not all of the oligomers falling within the general description given hereinabove will be equally suitable for use in the invention, and/or for use in any specific application of the invention. However, the skilled person will be able to determine/identify the most suitable - and therefore most preferred - oligomers, for instance using one or more of the *in vitro* or *in vivo* assays described in the Experimental Part, or any other suitable *in vitro* or *in vivo* assay. E.g. a selection of suitable oligomers may be made by means of the ability of the cells to bind to HLA-A2.1 molecules, e.g. as determined by peptide-induced HLA-A2.1 upregulation (Nijman, H. W., et al., Eur.J.Immunol., 23 : 1215-1219, 1993; and further

described in Examples III and IX) and/or in the competition-based HLA-A2.1 peptide binding assay of by van der Burg et al. (van der Burg, S. H., et al., Hum. Immunol. 44. 44(4. 4), 189-98. 1995; and further described in Example IV).

Usually, oligomers suitable for use in the invention will show an ability to upregulate HLA-A2.1 in the HLA-A2.1 upregulation assay of Nijman et al. Preferably, in this assay, the ability of the oligomer to upregulate HLA-A2.1 will be at least equal to, and most preferably be greater than, the corresponding ability of the reference oligomer YLEPGPVTA (gp100:280-288), which may be used as a positive control.

Furthermore, in the competitive binding assay of van der Burg, a preferred oligomer of the invention will usually show a binding affinity that - on the basis of the determined IC50-value - can be classified as "intermediate" (i.e. an IC50-value of between 5 μM and 15 μM) or as "high"(i.e. an IC50-value of < 5 μM).

The oligomers may also be tested for their ability to induce a CTL response *in vivo*, i.e. using in a suitable *in vivo* assay, such as the *in vivo* assay using HLA-A2.1 Kb transgenic mice described in more detail in the Experimental Part hereinbelow, vide in particular Examples VI and X. In such an assay, an oligomers of the invention should most preferably be able to induce a CTL response *in vivo*.

Also, when the lytic activity of the oligomer-specific CTLs thus generated in the transgenic mice are determined, for instance using the chromium release assay of Bakker et al. (Bakker, A. B. H., et al., J.Exp.Med., 179: 1005-1009, 1994.) further described in Example V hereinbelow, the cytotoxic T cells induced by the oligomer - when used as effector cells in this assay, with for instance cells of the tumor to be treated being used as the target cells - should be able to lyse the target cells, and most preferably should be able to provides a lytic activity (expressed as the percentage of specific lysis) that is at least 2 times the background level of induction of specific effector cells, i.e. compared to the antigen negative cells.
On the basis of these assays, the (oligo)peptide of Formula I (HLSTAFARV) was identified as one of the most preferred oligomers of the invention.

The oligomers of the invention can be prepared in a manner known per se, for instance via peptide synthesis techniques involving the use of protected amino acids, carbodiimide chemistry and/or Fmoc chemistry, which may be carried out using automated equipment. Other techniques include, but are not limited to, solid-phase peptide synthesis, for instance as described by Merrifield, J.Am.Chem.Soc., 85, 2149 (1963). These and other techniques will be known to the skilled person and/or are described in the standard handbooks.

If the (amino acid residues present in) the oligomers of the invention carry basic or acidic groups, the oligomers may be prepared and/or used in the invention as their pharmaceutically acceptable salts.

The invention is based on the discovery that the (oligo)peptides of SEQ ID no.1 and their analogues, and in particular the nonapeptide of Formula I and the (oligo)peptide analogues derived therefrom, can be used to illicit *in vivo* an immune response against immunogenic tumors. Furthermore, it has been found that the immune response provided/generated by the oligomers of the invention is highly specific for immunogenic tumors, and in particular for renal cell carcinoma, in that it "attacks" the tumor (cells) essentially without generating an immune response against other cells, parts, tissues or organs of the body.

To generate the immune response, an oligomer of the invention is administered to the body of a human or animal in such a way that it is or may be presented to (the cells involved in) the immune system, i.e. in such a way that an immune response, in particular a significant immune response, more in particular a therapeutically effective immune response is generated. Generally, this will involve a route of administration such that, upon administration, the oligomer may come into contact with suitable antigen presenting cells - including but not limited to dendritic cells, B-cells and/or peripheral blood lymphocytes - in such a way that the oligomer may be "picked up" by the antigen presenting cells. The antigen presenting cells may then present the oligomer to other cells of the immune system involved in generating an immune response, such as T cells, to provide the desired immune response. This may for instance comprise a primary immune response and/or a secondary immune response, including but not limited to production of antibodies and/or induction of antigen-specific cytotoxic T-cells.

Usually, according to the invention, the oligomer is administered to a part, tissue and/or organ of the body where the oligomer may come into contact with antigen presenting cells. For instance, it is known that dendritic cells are present in the skin. One possible route of administering the oligomers of the invention therefore comprises subcutaneous and/or intradermal administration. Other suitable routes of administration include, but are not limited to, intravenous and/or intralymphatic administration.

Another technique for administering the oligomers to the body of a patient comprises administration "*in conjunction with an antigen presenting cell*", by which is meant that the oligomer is administered while carried by, attached to or otherwise associated with a suitable antigen presenting cell. For this purpose, a suitable antigen presenting cell may be

"loaded/primed" *in vitro* with an oligomer of the invention, i.e. prior to administration, for instance by adding the oligomer to a solution, preparation or *in vitro* culture of the antigen presenting cell(s). The antigen presenting cells thus loaded with the oligomer are then administered to the body of a patient, e.g. via intradermal injection, upon which they may (further) illicit an immune response against the oligomer, e.g. by presenting the oligomer to T-cells. Preferably, in this embodiment, antigen presenting cells are used that have been obtained from the patient - i.e. in a manner known per se - or that have been derived from (e.g. cultured from) antigen presenting cells obtained from the patient. Preferably, dendritic cells are used, although the invention is not limited thereto.

For either of the above modes of administration, the oligomer of the invention should most preferably be such that it can be "picked up" - either *in vivo* or *in vitro* - by the antigen presenting cell(s) in a way that allows the antigen presenting cell(s) to present the oligomer to further cells involved in generating the desired immune response, such as the T-cells. For this, it is usually required that the oligomer can bind, adhere or otherwise attach itself to the antigen presenting cells, and in particular to the pertinent antigen-receptors on the surface of the antigen presenting cells, such as the major histocompatiblity complex molecules. Usually, this means that the number of amino acid residues of the (oligo)peptide of SEQ ID no.1 or the analogue thereof should be as mentioned above and in particular be 8-10, more in particular be 9 amino acid residues. The most preferred embodiment of the invention therefore comprises the use and/or the administration of an octa-, nona- or decapeptide of the invention, and in particular of a nonapeptide. However, also included within the scope of the invention is the use – e.g. administration – of a precursor of an oligomer of the invention, and in particular of a precursor of an octa-, nona-, or decapeptide of the invention, although the use of such precursors is usually not required. A precursor of an oligomer of the invention is herein understood to mean any molecule which upon processing is capable of producing an oligomer of the invention.

According to the invention, treatment of a patient usually involves one or more administrations of an oligomer of the invention, in an amount sufficient for an immune response against the oligomer to be generated. Preferably, these administrations (also referred to as "*vaccinations*") with the oligomer are such that they result in a significant immune response as defined above, more in particular a therapeutically effective immune response as defined above. Generally, such a treatment will involve one or more "priming" administrations of the oligomer, followed by one or more "booster" administrations of the oligomer, the latter usually after a suitable period of time after priming, and - if several

booster administrations are used - separated by suitable time intervals. For instance, a suitable administration regimen may involve a first priming administration on day one, optionally followed by one or more further priming administrations in the next 8 weeks, in which said administrations may for instance be separated by 14 to 28 days or more. These priming administrations may then be followed by one or more booster administrations, e.g. in weeks 8 to 52, which booster administrations will usually be separated by 14 to 28 days or more. However, the invention is not limited to any specific administration regimen, nor to any specific combination of priming and boosting administrations. Any administration regimen that provides an immune response, more specifically a significant immune response, and even more specifically a therapeutically effective immune response, is incorporated within the scope of the invention.

The amount of oligomer administered during each vaccination (i.e. as a priming administration or as a booster administration) will be such that it results in an immune response, preferably a significant immune response, and more preferably a therapeutically effective immune response. On the basis of the description given herein, the clinician will be able to determine suitable amounts. Depending on the oligomer used, these will usually be in the region of 1 to 1000 microgram, preferably 50 to 250 microgram, for each of the one or more priming administrations, and in the region of 1 to 1000 microgram, preferably 50 to 250 microgram, for each of the one or more booster administrations (if any); in which the same or different amounts of antigen may be administered during each of the priming and/or booster administrations. For instance, a suitable regimen may involve a first priming administration using 250 microgram of antigen, followed by a further priming using 50 microgram of antigen; and one or more booster administrations using 50 microgram of antigen, e.g. according to a regimen as described above.

Usually, the one or more priming and the one or more booster administrations are administered via the same route of administration (e.g. subcutaneously and/or intradermally) and using the same (type of) pharmaceutical formulation for the oligomer (e.g. injectable solution), although the invention is not limited thereto. Also, usually only one (type of) oligomer of the invention will be used, although the invention also encompasses the use of any suitable combination of the oligomers described above, in any suitable manner.

The oligomers of the invention may be formulated in any suitable manner known per se, which preferably provides a pharmaceutical preparation that can be used to administer the oligomer(s) via one of the routes described above, so as to allow the oligomer to be presented to the immune system. Usually, this will involve formulating the oligomers as such or as a

pharmaceutically acceptable salt, so as to provide a pharmaceutical form known per se, such as a tablet, a capsule, a powder, a freeze dried preparation, a solution for injection, etc.; optionally using one or more pharmaceutically acceptable carriers and/or adjuvants known per se. Injectable solutions, as well as freeze dried powders or other pharmaceutical forms that can be dissolved in, mixed with or reconstituted using a suitable liquid carrier to provide an injectable form, are usually preferred.

The pharmaceutical preparations of the invention will usually contain a predetermined amount of antigen, and preferably a unit dose of antigen, which may be in the region of 1 to 1000 microgram, preferably 50 to 250 microgram, of antigen per dose. The compositions of the invention may also be packaged, for instance in vials, ampoules, bottles, sachets, blisters, etc.; optionally with relevant patient information leaflets and/or instructions for use.

The oligomers of the invention may be administered in conjunction with (i.e. essentially simultaneously and/or in a combined administration regimen with) one or more other suitable or desired pharmaceutically acceptable substances, including but not limited to therapeutically active substances and substances for promoting or enhancing the immune response against the antigen. Examples of the latter are pharmaceutically acceptable immune adjuvants such as Freund's, saponin, ISCOM's, aluminium hydroxide or a similar mineral gel, or keyhole limpet hemocyanin or a similar surface active substance and/or immunogenic carriers such as CpG (cytidine phospho guanidine), administered in amounts known per se.

Examples of therapeutically active substance which may be administered in conjunction with the antigens of the invention include, but are not limited to, interleukines or interferons, Flt-3L. These may be co-administered in amounts known per se, which may also provide a synergistic effect.

The above immune adjuvants, therapeutically active substances, etc., may be administered separately or may be combined with the antigen to provide a combined preparation of the invention, which is preferably in a pharmaceutical form as described above. For instance, such a "combination preparation" of the invention may contain 1 to 1000 microgram, preferably 50 to 250 microgram of antigen in combination with a suitable amount of interleukine-2.

Again, the amount(s) of oligomer administered, the time (intervals) at which the oligomer is administered, the route of administration, the form in which the oligomer is administered, and/or the further substance(s) with which the oligomer is administered, may be determined by the clinician on the basis of the description given herein, taking into account such factors as the tumor to be treated, the general condition of the patient, the oligomer(s)

used, the type of vaccination (e.g. priming or boosting), and the desired administration regimen. It will also be clear to the skilled person that - as usual in the field of vaccinations/immunology - the immune response obtained need not necessarily be dose-dependant.

When an oligomer of the invention is administered in conjunction with an antigen presenting cell, i.e. as described above, the cells carrying the oligomer may be administered in a manner known per se for the administration of cells to the body of a patient. Again, this administration is preferably such that it results in an immune response, in particular a significant immune response, and preferably a therapeutically effective immune response. For instance, the cells carrying the oligomer may be administered via intradermal injection.

For this purpose, the cells are usually provided in the form of a cell suspension in a suitable liquid medium, which is most preferably pharmaceutically effective and which may be the same as the medium used for maintaining, cultivating and/or loading/priming the cells *in vitro*. Examples are water and physiological solutions, such as phosphate-buffer-salt-solutions. In such cell preparations for administration, the oligomer loaded cells will usually be present in amounts/concentrations of 10^2-10^8 cells/ml, preferably 10^4-10^5 cells/ml. Alternatively, the cells may for instance be provided/administered in the form of apoptotic bodies.

The cells can again be administered once or several times, for instance according to a regimen involving priming and boosting, e.g. essentially as described above, although the invention is not limited thereto. In doing so, the cells are preferably administered in suitable amounts, i.e. such that an immune response, in particular a significant immune response, and preferably a therapeutically effective immune response, is obtained. The specific amount(s) to be administered will usually be determined by the clinician on the basis of the description given herein, taking into account such factors as the tumor to be treated, the general condition of the patient, the cells and/or oligomers used, the type of vaccination (e.g. priming or boosting) and the desired administration regimen.

Furthermore, although not preferred, the invention also encompasses the administration of fractions, lysates and/or fragments that are obtained from cells that have been the loaded/primed *in vitro* with the oligomer, and in particular the cells described above. In particular those fractions, lysates or fragments may be used which - when administered in a suitable manner, in suitable amounts and/or according to a suitable regimen, e.g. as described above - can provide a significant immune response, and preferably a therapeutically effective immune response. Such cell fractions, lysates or fragments may be in the form of, or

incorporated into, a pharmaceutical preparation, which essentially may be as described above for the oligomers of the invention.

Finally, as mentioned above, precursors of the oligomers of the invention may be used. These will also preferably be such that - when administered in a suitable manner, in suitable amounts and/or according to a suitable regimen, e.g. as described above - they can provide a significant immune response, and preferably a therapeutically effective immune response. These precursors may again be formulated and administered essentially as described above for the oligomers of the invention.

The oligomers can be used in the treatment of neoplastic and pre-neoplastic diseases, such as cancer. In particular, they can be used in the treatment of immunogenic tumors, and more in particular of immunogenic tumors the cells of which contain or express on their surface the polypeptide of SEQ ID no.1 or at least an antigenic part or component thereof.

Examples thereof include, but are not limited to, renal cell carcinoma or other cancers of the kidneys, as well as cancer(s) of the prostate, the head and/or neck, the gastrointestinal tract or any part thereof, and/or the bladder. Of these, the oligomers of the invention can in particular be used in the treatment of renal cell carcinoma, as well as cancer(s) of the colon, stomach or bladder.

As such, the oligomers can be used to remove the tumor and/or reduce the size thereof, or at least prevent or limit the (further) growth of the tumor and/or the spread of a tumor (i.e. within the same part or organ of the body and/or to other parts or organs of body), including metastasis. Also, the oligomers may be used to prevent recidivism, for instance following surgical removal of the tumor.

In further aspects, the invention therefore relates to the use of an oligomer of the invention - i.e. an (oligo)peptide of SEQ ID no.1 or a variant and/or analogue thereof - in the preparation of a composition for the treatment of cancer, including but not limited to renal cell carcinoma or other cancers of the kidneys, as well as cancer(s) of the prostate, the head and/or neck, the gastrointestinal tract or any part thereof, and/or the bladder; and in particular in the preparation of a composition for the treatment of renal cell carcinoma, as well as cancer(s) of the colon, stomach or bladder. More specifically, the invention relates to the use of an oligomer of the invention in the preparation of a composition for the treatment of an immunogenic tumor, in particular of an immunogenic tumor that expresses the protein of SEQ ID no.1 or at least an immunogenic part thereof.

Besides the above therapeutic applications, the oligomers of the invention may also be used as antigens representative of the protein of SEQ ID no.1, for instance in immunological,

diagnostic and/or analytical applications, such as those described per se in WO 93/18152. For this purpose, the oligomers of the invention may also form part of a suitable kit, e.g. in combination with other suitable components for immunological, diagnostic and/or analytical kits.

Yet another aspect of the invention relates to a gene therapy agent that can be used to generate - by suitable administration to a human or animal (mammal) - a "significant" immune response as described above, and preferably a "protective" immune response as described above; which response is essentially similar to the significant/protective immune response that can be induced by administration of an oligomer of the invention as described above. Such a gene therapy agent will usually be such that, upon administration, it will allow or provide for expression of an (oligo)peptide of SEQ ID no. 1 - or a variant or analogue thereof as defined above - in the body of the human or animal, or in any part, organ, tissue or cell of such a human or animal, including (the cells of) the tumor to be treated. The expression of the oligomer provided by the gene therapy agent should further be such that the expressed oligomer can come into contact with an antigen-presenting cell as defined above, or otherwise can come into contact with cells involved in the immune system, so as to generate a significant, and preferably a protective, immune response against (at least) the oligomer. Usually, such a gene therapy agent of the invention will comprise a single or double stranded nucleotide sequence (e.g. a DNA or RNA sequence), which at least comprises a nucleotide sequence that codes for an oligomer of the invention. Usually, such a gene therapy agent will be in the form of a suitable vector – e.g. a viral vector such as an adenoviral vector - which at least encodes an oligomer of the invention. Such a vector may contain all other elements for gene therapy vectors known per se, including but not limited to genetic elements such as a suitable promoter, a suitable terminator or other regulatory elements operably linked to the oligomer-encoding sequence; as well as integration factors or other elements that allow for the gene therapy agent to enter into and be expressed in the cell, e.g. by integration into the (genomic) DNA present in the cell. Such a vector may also be suitably "packaged", e.g. with one or more suitable capsid proteins, to provide a viral particle. Also, such a gene therapy agent may express the oligomer of the invention as part of a larger amino acid sequence (e.g. a protein or polypeptide), or as a fusion with one or more further (oligo)peptide sequences.

The gene therapy agent may be administered in a manner known per se, e.g. by exposing the body of the human or animal - or any part, organ, tissue or cell of the human or animal, including (the cells of) the tumor to be treated - to the gene therapy agent. Also, such a gene therapy agent may be used in vitro to infect suitable cells - such as cells derived from

the patient and/or of the tumor to be treated - upon which these cells may then be introduced to the body of the human or animal, to provide the desired significant immune response, and preferably a protective immune response, as defined hereinabove.

The gene therapy agent may also be formulated in a manner known per se, e.g. to provide a pharmaceutical preparation (gene therapy preparation), e.g. using one or more pharmaceutically acceptable carriers, adjuvants, and/or excipients. Such preparations form a further aspect of the invention.

Description of the figures

Figure 1: 1. HLA-A2.1 binding affinity of G250-derived peptides. Binding of peptides to HLA-A2.1 was determined in a competition assay at 4°C using mild acid-treated HLA-A2.1 positive JY cells. The binding capacity of the peptides is shown as the concentration of peptide needed to inhibit 50% of binding of the HBC:18-27 peptide (FLPSDC(-fl)FPSV). Data represent the mean of two independent experiments.

Figure 2: Immunogenicity of G250-derived peptides in HLA-A2.1Kb transgenic mice. CTLs isolated from immunised mice were tested for lytic activity using chromium-labelled EL-4/HLA-A2.1Kb target cells loaded with relevant peptide (closed circles) or irrelevant peptide (open circles). One representative experiment is shown, in upper left corner the number of mice (positive/tested) is indicated.

Figure 3: Peptide G250:254-262 is naturally processed in HLA-A2.1Kb transgenic mice. Peptide-specific CTLs against peptide G250:254-262 were tested for their ability to lyse chromium-labelled EL-4/HLA-A2.1Kb target cells endogenously expressing G250 (closed circles), as a negative control EL-4/HLA-A2.1Kb target cells were used (open circles). Data representative of all mice tested is depicted.

Figure 4: Specific reactivity of DC-induced human CTLs against G250-derived peptides in vitro. A, Chromium-labelled HLA-A2.1 positive T2 target cells were loaded with relevant peptide (closed bars) or irrelevant peptide (open bars). As effector cells three independent CTL cultures were used. B, Peptide-specific CTLs reactive against G250:26-35, G250:254-262 and G250:421-429 were tested for their ability to lyse chromium-labelled BLM target cells endogenously expressing G250 (closed bars) and chromium-labelled BLM/gp100 target cells (open bars). Two independent G250:254-262 specific CTL cultures specifically lysed BLM/G250 cells.

Examples

In the Examples, the following abbreviations will be used: CTL, cytotoxic T lymphocyte; DC, dendritic cell; GM-CSF, granulocyte macrophage colony stimulating factor; LPS, lipopolysaccharide; MCM, monocyte conditioned medium; PBMC, peripheral blood mononuclear cells; RCC, renal cell carcinoma; TIL, tumor-infiltrating lymphocytes.

Example I: Synthesis of the oligomers

The oligomers were synthesised by Fmoc chemistry using a multiple peptide synthesiser. As determined by reversed phase high-performance liquid chromatography (HPLC) the peptides were > 90% pure. Peptides were dissolved in DMSO at a concentration of 10 mM, and stored at -20°C. Unlabeled and fluorescein-labeled peptides were obtained from J.W. Drijfhout (LUMC, Leiden, The Netherlands).

Example II: Cell culture

JY, T2, and autologous EBV-B cells were cultured in Iscoves medium enriched with 5% FCS and antibiotic-antimycotic (all obtained from Gibco, Grand Island, NY). The melanoma cell line, BLM expressing HLA-A2.1 (Bakker, A. B. H., et al., J.Exp.Med., 179: 1005-1009, 1994) was transfected with either the cDNA of G250 or gp100 resulting in the cell line BLM-G250 and BLM-gp100, respectively. The transfected cell lines BLM-G250 and BLM-gp100 were cultured in Dulbecco's modified eagle medium (DMEM, Gibco) enriched with 5% FCS and antibiotic-antimycotic in the presence of G418 (1 mg/ml, Gibco).

The mouse cell line EL-4 (ATCC) was transfected with a DNA construct encoding for the HLA-A2.1Kb chimeric molecule (Vitiello, A., et al., J.Exp.Med., 173: 1007-1015, 1991) with and without a DNA construct encoding G250 (EL-4/HLA-A2.1Kb/G250 and EL-4/HLA-A2.1Kb, respectively). These cell line were maintained in Iscoves with 5% FCS, G418 (1 mg/ml) and hygromycin (0.5 mg/ml).

Example III: HLA-A2.1 upregulation on T2 Cells

To determine whether the G250-derived peptides were able to bind to HLA-A2.1 molecules, peptide-induced HLA-A2.1 upregulation on TAP-deficient TxB cell hybrid T2 cells (Salter, R. D., et al., Immunogenetics., 21: 235-246, 1985) was examined as published previously (Nijman, H. W., et al., Eur.J.Immunol., 23 : 1215-1219, 1993). Briefly, 105 T2 cells were incubated together with various concentrations G250-derived peptides (final DMSO concentration 0.5%) for 14 hours at 37°C and 5% CO2 in serum-free Iscoves medium

in the presence of 3 μg/ml human ß2-microglobulin (Cymbus Biotechnology Ltd.). Upregulation of HLA-A2.1 molecules on T2 cells was analysed by flow cytometry using anti-HLA-A2 mAb BB7.2 (Parham, P. and Brodsky, F. M., Hum.Immunol., 3: 277-299, 1981.). The fluorescence index (FI) was expressed as: [experimental mean fluorescence ÷ background mean fluorescence]-1. Mean values for background fluorescence were measured by incubating T2 cells with an HLA-A2.1 non-binding peptide at similar concentrations.

Example IV: Competition-based HLA-A2.1 peptide-binding assay

The binding affinity of the peptides for HLA-A2.1 molecules was examined as described by Van der Burg et al. (van der Burg, S. H., et al., Hum Immunol 44. 44(4. 4), 189-98. 1995.). Briefly, HLA-A2.1 molecules on JY cells were stripped of peptides by treatment with ice-cold citric acid buffer (pH 3.2, 90 sec). After washing, the JY cells were incubated with 150 nM of fluorescein-labeled reference peptide (FLPSDC(-fl)FPSV) and different concentrations of competitor peptide in the presence of 1 μg/ml ß2-microglobulin (Cymbus Biotechnology Ltd.,) for 24 hours at 4°C. Cells were washed, fixed and fluorescence intensity was analyzed by flow cytometry. The inhibition was calculated using the formula: [1-[mean fluorescence (MF) reference & competitor peptide - MF no reference peptide] ÷ [MF reference peptide - MF no reference peptide]] x 100%. Binding affinity of the competitor peptides was determined as the concentration needed to inhibit 50% of binding of the fluorescein-labeled reference peptide (IC50). Binding affinity was categorised as: high IC50 < 5 μM, intermediate > 5 μM IC50 < 15 μM and low IC50 >15 μM. Preferably, in this assay, an oligomer of the invention has an intermediate or high binding affinity.

Example V: Cytotoxicity assay

CTL activity was measured using the chromium release assay as described previously (Bakker, A. B. H., et al., J.Exp.Med., 179: 1005-1009, 1994). Briefly, 106 target cells were incubated with 100 μCi Na2[51Cr]O4 (Amersham, Bucks, UK) for 45 min at 37°C. After extensive washing, chromium-labelled target cells (2*104 cells/ml) were loaded with 10 μM peptide for 30 min at 37°C. Effector cells were added to 103 target cells in triplicate wells of a round bottom microtiter plate (total volume 150 μl). After a 4-hour incubation, 100 μl of the supernatant was harvested and its radioactivity content was measured. The specific chromium release was defined by: [experimental release - spontaneous release (SR) ÷ maximum release - SR] x 100%.

Example VI: In vivo assay using transgenic mice

HLA-A2.1Kb transgenic C57BL/6 mice were kindly provided by Dr. L. Sherman (Scripps Laboratories, San Diego, CA). Mice were held under specified pathogen-free conditions at the Central Animal Laboratory, Nijmegen University (Nijmegen, The Netherlands). For experimental purposes, mice at the age of 8-12 weeks were used. HLA-A2.1Kb expression (anti-HLA-A2 mAb BB7.2) was analyzed by flow cytometry on peripheral blood mononuclear cells (PBMC) isolated from 4 weeks old mice.

Groups of 3 mice were vaccinated twice subcutaneously in the flank with 0.1 μmol of G250-derived peptide and HBV core antigen-derived T helper epitope emulsified in Incomplete Freund's Adjuvant (Difco Lab., Detroit, USA) with a two week interval. Mice were sacrificed 14 days after the last immunisation, and spleen cells (40*106 in 10 ml) were restimulated with peptide-loaded syngeneic irradiated lipopolysaccharide (LPS)-stimulated B cell lymphoblasts (ratio 4:1). Cells were cultured in Iscoves medium supplemented with 5% FCS, antibiotic-antimycotic and 50 μM ß-mercaptoethanol. Six days after restimulation, responder T cells were harvested and tested for specific cytolytic activity.

Example VII: In vitro induction of human CTLs using peptide-loaded DCs

PBMC of healthy individuals were separated using Percoll-density centrifugation and were allowed to adhere for 1 hour at 37°C in RPMI-1640 (Gibco) enriched with 2% HS in 6-well tissue culture plates (Costar, Badhoevedorp, The Netherlands). Adherent monocytes were cultured in RPMI-1640 enriched with 10% FCS in the presence of IL-4 (500 U/ml, Schering-Plough, Amstelveen, The Netherlands) and granulocyte macrophage colony stimulating factor (GM-CSF, 800 U/ml, Schering-Plough) for 6 days. Fresh cytokine-containing culture medium was added at day 3. At day 6, the immature dendritic cells (DC; MHC class I positive and low expression of class II, B7.1 (CD80), B7.2 (CD86) and CD83) were cultured in the presence of 50% (v/v) monocyte-conditioned medium (MCM), obtained from IgG-activated monocytes (Romani, N., et al., J Immunol Methods 196. 196(2. 2), 137-51. 1996.). Mature monocyte-derived DCs, expressing MHC class I and II, B7.1 and B7.2 and CD83, were harvested at day 8. Mature DCs were pulsed with 50 μM peptide and 3 μg/ml ß2-microglobulin (37°C, 2 hours). Peptide-loaded and irradiated (2500 rad) DCs (2.5*105/well) were co-cultured with enriched autologous CD8 positive T cells (5*105/well, depleted for CD4 positive and CD56 positive cells by magnetic sorting (Dynal, Oslo, Norway)) in the presence of autologous irradiated PBL (5*105/well) and IL-7 (10 ng/ml; Genzyme,

Cambridge, MA) in a 24-well plate (Costar). One day after stimulation, IL-10 (20 U/ml; Dynax, Palo Alto, CA) was added to the cell cultures.

At day 7, responder T cells were restimulated with peptide loaded autologous irradiated immature DCs (2.5*105/well). IL-10 was added at day 1 and IL-2 (15 U/ml; Cetus Corp., Emeryville, CA) at day 2 and 5 after restimulation. Restimulation of the responder T cells at day 14 was performed with immature peptide-loaded DCs, IL-2 was added after 1 day. All wells containing responder T cells were kept separately and at day 21, half of the bulk CTLs of each well were used in a cytotoxicity assay with peptide-loaded T2 cells as target cells. Bulk CTLs showing peptide-specific cytotoxic activity were weekly stimulated with 15 U/ml IL-2 and tumor cells endogenously expressing G250.

Example VIII: Identification of HLA-A2.1 binding peptides derived from G250

The G250 protein is highly expressed in primary and metastatic RCC. The ability to induce CTLs against G250 could therefore be of great importance for treatment of RCC patients. Since HLA-A2.1 is a frequently expressed MHC molecule in Caucasians, the presence of HLA-A2.1-restricted CTL epitopes in the G250 antigen was investigated.

Based upon the sequence of the G 250 protein, 60 potential HLA-A2.1 binding peptides were selected and investigated. These G250-derived peptides were first tested for their ability to bind to HLA-A2.1 molecules using the HLA-A2.1 upregulation assay on TAP-deficient T2 cells. The T2 cells were incubated with 25 μM and 50 μM G250-derived peptide, 14 hours later the number of HLA-A2.1 molecules at the surface of the cells were measured using anti-HLA-A2.1 mAbs. From the 60 predicted peptides, 18 G250-derived peptides were found to be able to upregulate HLA-A2.1 molecules: these are shown in Table III.

Table III: HLA-A2.1 upregulation of G250-derived peptides with HLA-A2.1 binding motif.

		HLA-A2.1 upregulation[a]	
position	AA sequence	50 μM	25 μM
G250:12-21	LLIPAPAPGL	1.27[b]	1.14[b]
G250:26-35	LLSLLLLMPV	0.77	0.22
G250:31-40	LLMPVHPQRL	1.03	1.09
G250:107-116	SLKLEDLPTV	2.27	0.90
G250:169-177	QLAAFCPAL	1.59	1.18
G250:181-190	ELLGFQLPPL	1.44	1.44
G250:182-190	LLGFQLPPL	1.40	1.27
G250:205-213	TLPPGLEMA	0.76	0.51
G250:254-262	HLSTAFARV	0.64	0.51
G250:271-279	GLAVLAAFL	0.59	0.27
G250:291-299	QLLSRLEEI	1.24	0.99
G250:350-359	MLSAKQLHTL	0.55	0.47
G250:362-371	TLWGPGDSRL	0.67	0.39
G250:372-381	QLNFRATQPL	0.61	0.49
G250:384-392	RVIEASFPA	0.80	0.54
G250:409-418	CLAAGDILAL	1.14	1.13
G250:415-423	ILALVFGLL	0.51	0.09
G250:421-429	GLLFAVTSV	1.47	0.98
gp100:280-288[c]	*YLEPGPVTA*	*1.50*	*1.48*

Notes:

a. HLA-A2.1 upregulation was analyzed using the TAP-deficient T2 cell line at the indicated peptide concentrations. Numbers indicate fluorescence index (FI).

b. Data represent the mean FI of two independent experiments.

c. As a positive control peptide gp100:280-288 was used.

Example IX: Binding-affinity of the G250-derived peptides for HLA-A2.1 molecules

Using the previously described competition-based HLA-A2.1 peptide-binding assay (van der Burg, S. H., et al., Hum Immunol 44. 44(4. 4), 189-98. 1995.) the binding affinity of the 18 selected peptides to HLA-A2.1 molecules were determined. After mild acid elution, the JY cells expressing empty HLA-A2.1 molecules were incubated with titrated concentrations of G250-derived peptides and a standard concentration of a fluorescein-labeled reference peptide having a high binding affinity to HLA-A2.1 molecules. The concentration of the G250-derived peptides which inhibited the binding of the reference peptide by 50% (IC50) is shown in Figure 1.

From the 18 tested G250-derived peptides, 4 peptides with a high (IC50 < 5 μM), 9 peptides with an intermediate (> 5 μM IC50 < 15 μM) and 5 peptides with a low binding affinity (IC50 > 15 μM) were found. The G250-derived peptides with high and intermediate affinity for HLA-A2.1 molecules were further studied to determine their immunogenicity in vivo and in vitro.

Example X: Immunogenicity of G250-derived peptides in HLA-A2.1Kb transgenic mice

HLA-A2.1Kb transgenic mice were used to investigate whether the aforementioned G250-derived peptides were able to induce a CTL response in vivo. These transgenic mice express the chimeric HLA-A2.1Kb molecule, in which the α3 domain of HLA-A2.1 is replaced by the corresponding murine Kb α3 domain. This results in a MHC molecule that can present peptides binding to the human HLA-A2.1 molecule and is still capable to interact with the CD8 molecule present on murine T cells. Spleen cells of HLA-A2.1Kb mice vaccinated with G250-derived peptide were restimulated once in vitro and used in a cytotoxicity assay. In this assay EL-4/HLA-A2.1Kb cells loaded with relevant G250-derived peptide were used to measure peptide-specific lysis of bulk CTLs and the same cells loaded with irrelevant peptide were used to measure non-specific lysis.

As can be seen from the results shown in Table IV, of the 13 G250-derived peptides investigated, 4 were found to be able to induce CTLs with peptide specificity in bulk cultures.

Table IV: Immunogenicity of G250-derived peptides in vivo and in vitro

		immunogenicity	
position	AA sequence	*in vivo*[a]	*in vitro*[b]
G250:12-21	LLIPAPAPGL	0/3	0/2
G250:26-35	LLSLLLLMPV	3/3	½
G250:107-116	SLKLEDLPTV	0/3	0/2
G250:169-177	QLAAFCPAL	0/3	0/2
G250:181-190	ELLGFQLPPL	0/3	0/2
G250:182-190	LLGFQLPPL	1/3	0/2
G250:254-262	HLSTAFARV	2/3[c]	2/3[c]
G250:271-279	GLAVLAAFL	3/3	0/2
G250:291-299	QLLSRLEEI	0/3	0/2
G250:384-392	RVIEASFPA	0/3	0/2
G250:409-418	CLAAGDILAL	0/3	0/2
G250:415-423	ILALVFGLL	0/3	0/2
G250:421-429	GLLFAVTSV	3/3	½

Notes:

a. Peptide-specific bulk CTLs induced in HLA-A2.1Kb transgenic mice, number of mice (positive/tested).

b. Peptide-specific bulk CTLs against G250-derived peptides using DCs of healthy donors, number of healthy donors (positive/tested).

c. Endogenously processed G250 recognised by bulk CTLs.

As shown in Figure 2, the high binding affinity peptide G250:26-35 and the intermediate binding affinity peptides G250:254-262, G250:271-279 and G250:421-429 were able to induce a specific bulk CTLs response.

To address whether these immunogenic epitopes are naturally processed and presented, EL-4 cells stably transfected with cDNAs encoding HLA-A2.1Kb and G250 were used as target cells in a chromium release assay using the 4 peptide-specific CTLs as effector cells. As shown in Figure 3, only CTLs directed against the peptide G250:254-262 was able to specifically lyse EL-4 cells expressing both HLA-A2.1Kb and G250. This indicates that the G250-derived peptide 254-262 is both an immunogenic and a naturally processed CTL epitope in HLA-A2.1Kb transgenic mice.

Example XI: Induction of human CTL responses against G250 in vitro

To investigate the immunogenicity of the aforementioned 13 G250-derived peptides in humans, they were analyzed for their ability to induce a CTL response in vitro. Each peptide was tested at least twice, using PBMC from different donors. For this purpose, monocytes of healthy HLA-A2.1 positive donors were cultured in the presence of GM-CSF and IL-4 to generate immature DCs. After maturation with MCM, DCs were loaded with G250-derived peptides and co-cultured with autologous CD8 positive T cells. After restimulating responder T cells twice with peptide-loaded immature DCs, the cytoxicity of these bulk CTLs were tested against T2 cells loaded with either the relevant G250-derived peptide or an irrelevant peptide (Table IV). As shown in Figure 4a, DCs loaded with peptides G250:26-35, G250:254-262 or G250:421-429 were all capable of inducing peptide-specific bulk CTLs.

To investigate whether any of these peptides are naturally processed and presented, peptide-specific CTLs were tested for their ability to lyse target cells endogenously expressing G250. As observed in HLA-A2.1Kb transgenic mice, two independent bulk CTLs cultures against peptide G250:254-262 were able to specifically lyse HLA-A2.1 positive BLM cells transfected with the G250 cDNA but not BLM cells transfected with gp100 cDNA (Figure 4b). In contrast, the CTLs induced against peptides G250:26-35 and G250:421-429 did not specifically lyse the BLM cells endogenously expressing the G250 antigen as the same background lysis was observed on BLM/gp100 transfectants (Figure 4b).

Together, these data demonstrate that the G250-derived peptide 254-262 is not only immunogenic in HLA-A2.1Kb transgenic mice but that also in humans CTLs capable of specifically lysing cells endogenously presenting this G250-derived T cell epitope can be obtained.

Hereinabove, G250-derived peptides were examined for their capacity to bind HLA-A2.1 molecules using an HLA-A2.1 upregulation assay and a competition-based HLA-A2.1 peptide-binding assay. Of the 60 peptides studied, four G250-derived peptides with high and nine peptides with intermediate binding affinity for HLA-A2.1 molecules were defined.

Also, using HLA-A2.1Kb transgenic mice and DCs of healthy HLA-A2.1 positive donors, it was demonstrated that CTLs could be induced against at least one G250-derived peptide, i.e. G250:254-262, which specifically lysed tumor cell lines positive for G250 and HLA-A2.1Kb or HLA-A2.1, respectively. This demonstrates that G250 contains an immunogenic epitope of amino acid 254-262 that is both endogenously processed and presented via HLA-A2.1.

This amino acid sequence from 254 to 262 (HLSTAFARV) - a MHC class-I-restricted T cell epitope within the RCC-associated antigen G250 - is a CTL epitope that is both naturally processed and presented in the context of the HLA-A2.1 molecule.

In addition to the naturally processed and presented G250:254-262 epitope, it was found that two other G250-derived peptides, G250:26-35 and G250:421-429, gave rise to peptide-specific CTLs both in vivo and in vitro.

One immunogenic peptide (G250:271-279) gave rise to peptide-specific CTLs using HLA-A2.1Kb transgenic mice and not using DCs of healthy donors. Possibly, this discrepancy can be explained by the fact that G250 is a self-antigen in humans and as a consequence T cells recognising this particular epitope may be deleted in the human T cell repertoire.

Three G250-derived peptide-specific bulk CTLs were not able to lyse cells endogenously expressing G250. It is possible that at least some of the peptides are either not properly processed by the proteasome or not properly transported by the TAP molecules .

The above also shows that the reversed immunology approach is a potent method to identify CTL-restricted epitopes in tumor antigens. Also, G250 is a novel target that is recognised by CTLs.

The G250 antigen is expressed in a high percentage of RCC and less in some other carcinomas. G250 is neither expressed in normal kidney or in testis. In addition, the G250 antigen is not mutated as no sequence differences were found between an RCC-derived G250 cDNA and the G250/MN/CA IX gene characterised from a healthy individual. Weak cytoplasmic staining of some gastric mucosal cells and cells of the larger bile-ducts by the

G250 mAb has been noted, suggesting that the G250 antigen may be an overexpressed differentiation antigen.

Together with the high prevalence of G250 in RCC patients, these above results underline the usefulness of G250-based oligomers in the development of immunotherapies for RCC and other G250 expressing tumors.

CLAIMS

1. A peptide containing a sequence of 6-15 contiguous amino acids from the amino acid sequence of SEQ ID no.1, or a variant of the peptide with at most 3 amino acid replacements with respect to the amino acid sequence of SEQ ID no.1.

2. A peptide according to claim 1, wherein the peptide contains an amino acid sequence that is chosen from the group consisting of SEQ ID no. 2 - 14, and variants thereof with no more than 3 amino acid replacements.

3. A peptide according to claim 1 or 2, wherein the peptide contains an amino acid sequence that is chosen from the group consisting of SEQ ID no. 2, SEQ ID no. 4, SEQ ID no. 9, and SEQ ID no. 14, and variants thereof with no more than 3 amino acid replacements.

4. A peptide according to any of claims 1-3, wherein the peptide contains the amino acid sequence of SEQ ID no. 2, or a variant thereof with no more than 3 amino acid replacements.

5. A pharmaceutical composition comprising a peptide as defined in any one of claims 1-4, and a pharmaceutically acceptable carrier.

6. A composition comprising an antigen presenting cell, wherein the antigen presenting cell is loaded with a peptide as defined in any one of claims 1-4.

7. A composition according to claim 6, wherein the antigen presenting cell is a dendritic cell.

8. A composition according to claim 6 or 7, wherein the antigen presenting cell is a human cell.

9. Use of a peptide as defined in any of claims 1-4, in the preparation of a composition for the treatment of cancer.

10. Use according to claim 9, wherein the cancer is renal cell carcinoma, or a cancer of the kidney, the prostate, the head, the neck, the gastrointestinal tract or any part thereof, or the bladder.

11. Use according to claim 10, wherein the cancer is renal cell carcinoma, or a cancer of the colon, stomach or bladder.

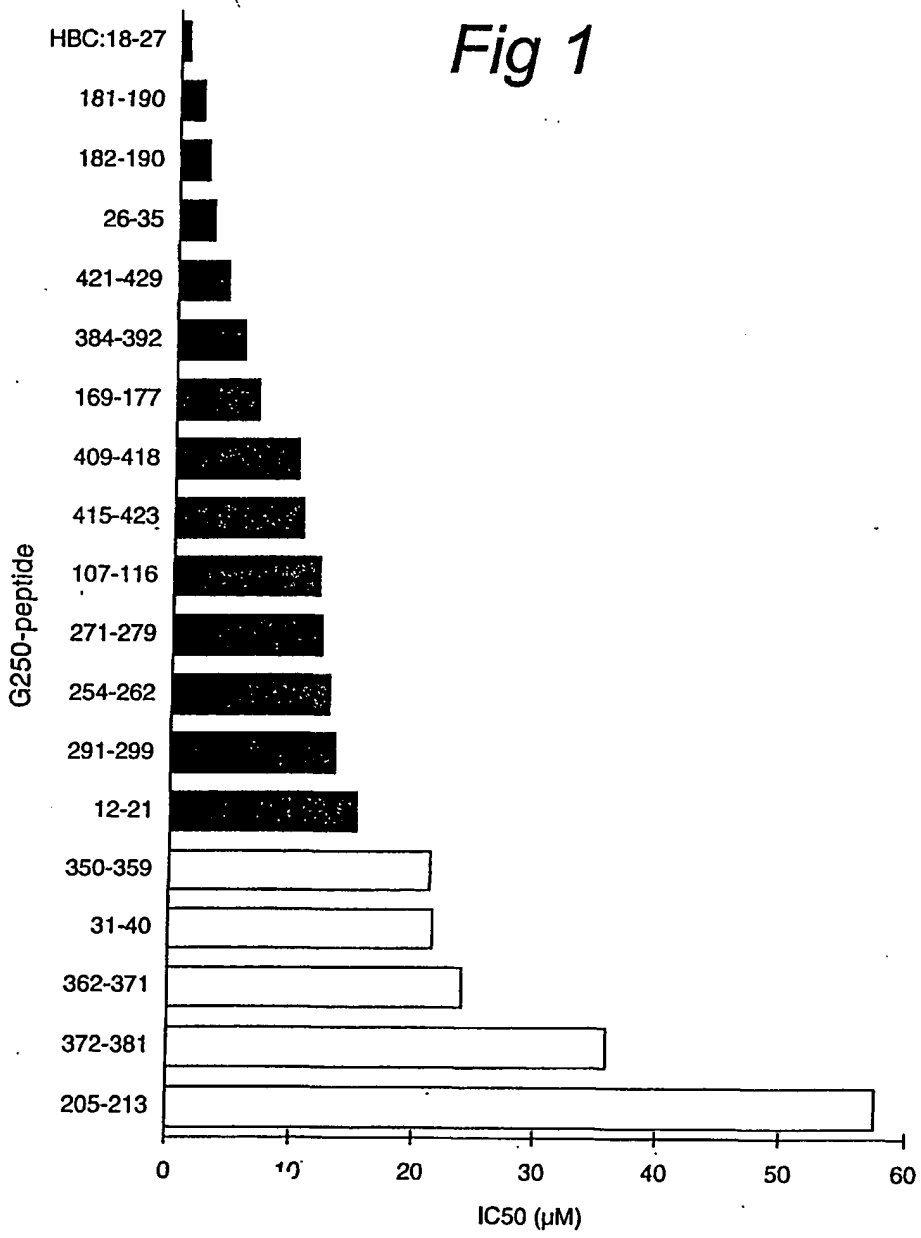
12. Use of a peptide as defined in any one of claims 1-4, in the preparation of a composition for the treatment of an immunogenic tumor that expresses the protein of SEQ ID no.1 or at least an immunogenic part thereof.

13. Use of a peptide as defined in claim 4, in the preparation of a composition for the treatment of cancer.

14. Use of a peptide as defined in claim 4, in the preparation of a composition for the treatment of an immunogenic tumor that expresses the protein of SEQ ID no.1 or at least an immunogenic part thereof.

15. A gene therapy agent, comprising a nucleotide sequence that at least encodes a peptide as defined in any one of claims 1-4, and optionally one or more further elements of gene therapy agents known per se.

Fig 1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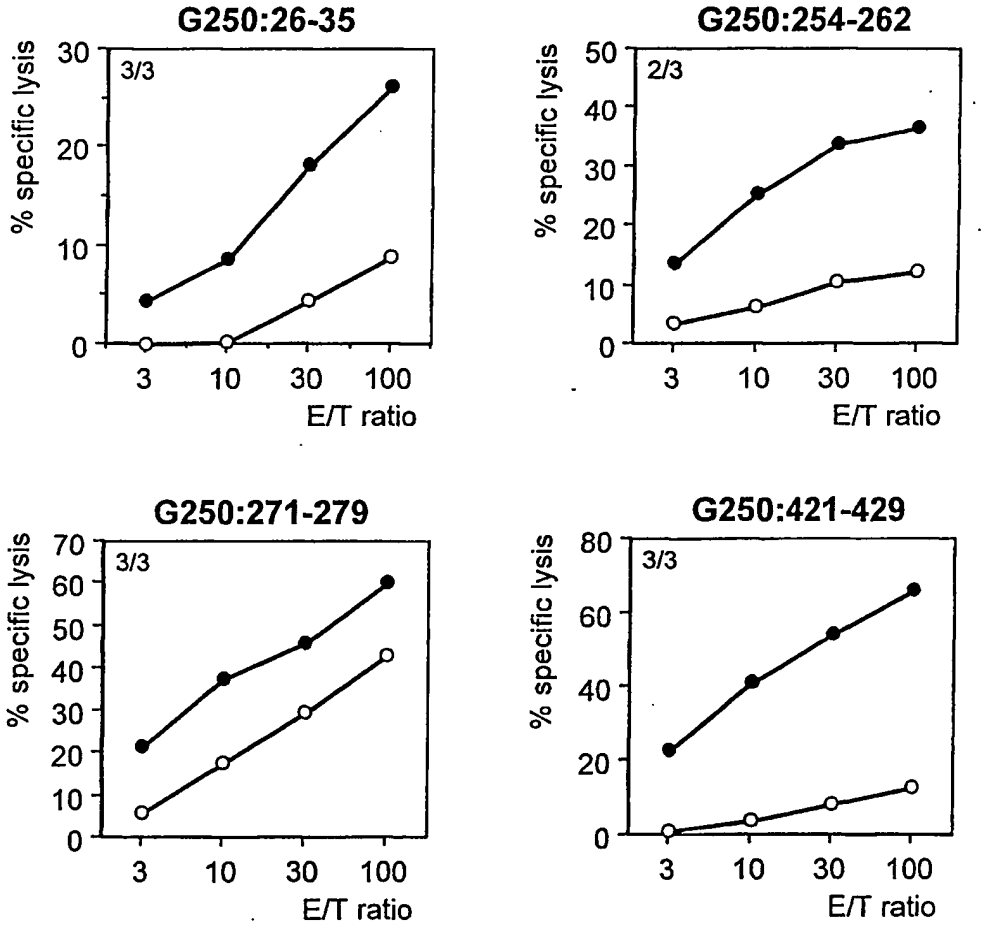
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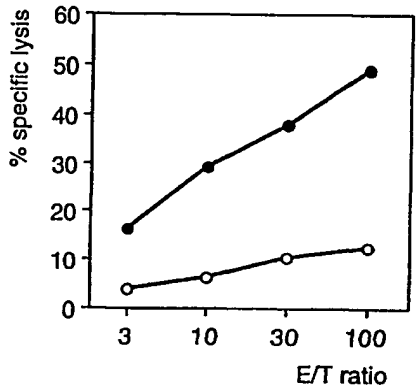

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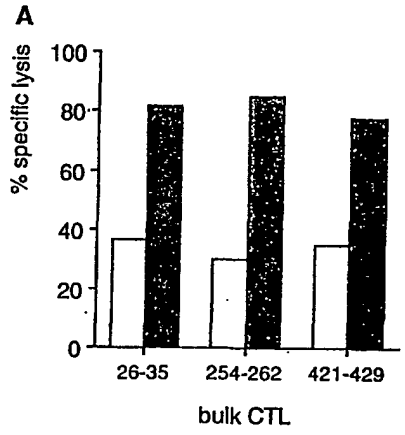

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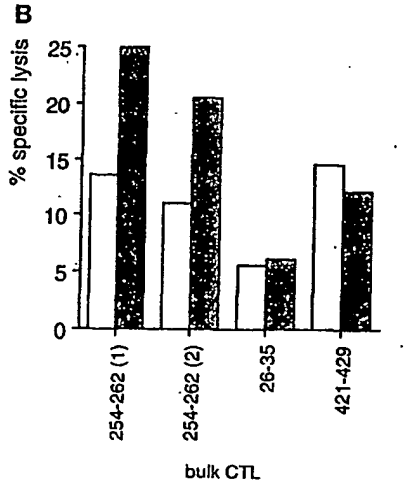

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